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   Patents ADP number *(if you know it)*

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4. Title of the invention

   COMPOUNDS

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This invention relates to antisense oligodeoxynucleotides targeted to sequences in thymidylate synthase (TS) mRNA. In particular the invention relates to antisense oligodeoxynucleotides targeted to sequences in the 3' end of TS mRNA, which antisense oligodeoxynucleotides are both cytostatic on their own when administered to human tumour cell lines, and also enhance the toxicity of the anticancer drugs such as Tomudex administered to those cells. In contrast, antisense oligodeoxynucleotides targeted to sequences at or near the translation start site at the 5' end of TS mRNA have either no effect, or enhance cell growth, when administered on their own. In addition, antisense nucleic acids targeted to these 5' sequences (but not to 3' sequences) induce TS gene transcription. The invention also relates to a combination product comprising an antisense oligodeoxynucleotide in combination with an anticancer agent such as Tomudex (N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl)-L-glutamic acid) or the Zeneca development compound ZD 9331 ((S)-2-(2-fluoro-4-[N-(4-hydroxy-2,7-dimethylquinazolin-6-ylmethyl)-N-(prop-2-ynyl)amino]benzamido)-4-(1H-1,2,3,4-tetrazol-5-yl)butyric acid), and to the use of such a combination product in the treatment of cancer.

Thymidylate synthase (TS) (EC 2.1.1.45) catalyses the conversion of deoxyuridylate to thymidylate, and is a housekeeping enzyme essential for the only intracellular *de novo* synthesis of thymidylate (Danenberg, 1977). TS gene expression is tightly regulated with respect to cell proliferation state (Maley and Maley, 1960; Lochsin *et al.*, 1979). As such, the TS gene is part of a group of genes whose expression is elevated at the G_1/S cell cycle boundary, and it has been suggested that transcription of several S-phase genes (including dihydrofolate reductase and thymidine kinase) is controlled in part by the E2F family of transcription factors (Farnham *et al.*, 1993; Mudrak *et al.*, 1994). In fact, transfection of active E2F1 genes into mouse cells induces expression of TS and other S-phase and cell cycle-regulated genes (De Gregori *et al.*, 1995). As cells progress through the cell cycle from G_0 through S phase, TS mRNA levels increase approximately 20-fold and TS enzyme activity increases about 10-fold (Navalgund *et al.*, 1980). However, TS gene transcription rate is upregulated only 2 to 4 times, suggesting that post-transcriptional events play a major role in TS regulation (Ayusawa *et al.*, 1986; Jenh *et al.*, 1985; Johnson, 1994). Differences in TS

mRNA stability are not likely to be critical in regulation, as TS mRNA half-life is about 8 hours in both resting and growing rodent cells (Jenh *et al.*, 1985). On the other hand, TS mRNA translation appears to be regulated by the TS protein itself, which specifically interacts at two sites within its own mRNA to inhibit protein production (Chu *et al.*, 1991, 1993b, 1994; Voeller *et al.*, 1995). Translation of other mRNAs (including c-*myc* mRNA) may also be regulated by interactions with TS protein (Chu *et al.*, 1995).

Because of its role in DNA precursor synthesis, TS has been identified as a potential target for cancer chemotherapeutic agents (Hardy *et al.*, 1987). High TS levels have been correlated with poor prognosis in patients with ovarian cancer (Suzuki *et al.*, 1994), rectal cancer (Johnston *et al.*, 1994) childhood acute non-lymphoblastic leukaemia (Volm *et al.*, 1994), and non-small cell lung carcinoma (Volm and Mattern, 1992). However, its prognostic value is not high in all tumour types (Peters *et al.*, 1986, 1994). Two types of TS inhibitors have been developed: (a) nucleotide analogues (including 5-FU, its riboside, and deoxyriboside derivatives) which must be activated to 5-fluorodeoxyuridylate (FdUMP) within cells to be effective (Heidelberger *et al.*, 1983) and (b) 5,10-CH_2FH_4 (antifolate) analogues, including *N*-10-propargyl-5,8-dideazafolate (CB3717) (Calvert *et al.*, 1986) and Tomudex (ZD1694; *N*-[5-(*N*-[3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl]-*N*-methylamino)-2-thenoyl]-L-glutamic acid) (Jackman *et al.*, 1991a, 1991b). Although Tomudex and 5-FU inhibit TS and have potent cytotoxic and antitumour activity (Heidelberger *et al.*, 1983; Keyomarsi *et al.*, 1993), they have an unusual biochemical effect. When human cancer cell lines are treated with 5-FU or Tomudex, TS levels increase rapidly, perhaps as a result of the release of translational inhibition by the TS protein (Keyomarsi *et al.*, 1993; Chu *et al.*, 1990; Chu *et al.*, 1993a).

It has been speculated that the release of translational inhibition that accompanies binding and inactivation of TS by chemotherapeutic agents (including Tomudex and 5-FU) might be prevented by treating cells with agents that could replace the specific interaction between TS mRNA and TS protein, and inhibit translation (Keyomarsi *et al.*, 1993) but no such agents were described. In another speculative article it was hypothesised that antisense nucleic acids designed to both reduce the ability of TS mRNA to direct protein production,

d to interact with the TS protein binding site, may be useful in complementing the effectiveness of drugs targeted against TS (Rapaport *et al.*, 1992).

To this end we attempted to specifically down-regulate the expression of TS in human breast cancer (MCF-7) cells in two ways. First, we both transiently and stably transfected the cells with vectors expressing antisense RNA molecules directed to hybridise to three different regions of the TS mRNA. Targeted sequences were: (1) sequences participating in the formation of a putative stem-loop structure surrounding the translation start site, and immediately adjacent and 3' to that site (these sequences also participate in binding TS protein to modulate translation), (2) the exon1/exon2 boundary and (3) the 3' end of the mature cytoplasmic mRNA. Antisense TS RNA was expressed from these vectors (as assessed by northern blot analysis and a novel modification of the run-on transcription assay to measure antisense transcription against background constitutive TS gene expression) (Koropatnick *et al.*, 1997). Second, we transiently transfected cells with single-stranded oligodeoxynucleotides targeted to hybridise to: (a) the translation start site and sequences surrounding it, (b) a sequence proximal to the translation start site and participating in the putative stem-loop structure, and (c) the translation stop site near the 3' end of the mature cytoplasmic RNA.

The present invention is based on our discovery that antisense oligonucleotides targeted to different mRNA sequences can inhibit, enhance, or have no effect on thymidylate synthase activity and tumour cell growth. Furthermore, transcription of constitutive TS gene expression is stimulated in response to antisense RNA and oligonucleotides directed toward sequences at or near the mRNA translation start site, but not in response to sequences within the coding region or at and surrounding the translation stop signal.

In a first aspect of the invention we provide an antisense oligodeoxynucleotide which hybridises to a target nucleic acid sequence in thymidylate synthase and which selectively inhibits thymidylate synthase production in mammalian cells. Preferably the oligonucleotide is targeted to sequences at or near the translational stop site at the 3' end of the TS gene, which sequences lie in the region between bases 800 and 1600, using the sequence numbering described for human thymidylate synthase mRNA by Takeishi *et al.*, 1985. More preferably

the sequences lie in the region between bases 1000 and 1530. Most preferably the sequenc[redacted] lie in the region between bases 1030 and 1460.

In a second aspect of the invention we provide an antisense oligodeoxynucleotide which hybridises to a target nucleic acid sequence in thymidylate synthase and which selectively enhances thymidylate synthase production in mammalian cells. Preferably the oligonucleotide is targeted to sequences at or near the translation start site at the 5' end of the TS gene, which sequences lie in the region between bases 1 and 300, using the sequence numbering in Takeishi *et al.*, 1985. More preferably the sequences lie in the region between bases 50 and 200. Most preferably the sequencs lie in the region between bases 90 and 130.

An antisense oligodeoxynucleotide is an oligonucleotide which is designed to hybridise to a specific region of a target nucleic acid sequence. The target nucleic acid is the TS gene or mRNA transcribed from the TS gene. Preferably the target nucleic acid is the mRNA encoding thymidylate synthase.

The effects of antisense oligonucleotides on thymidylate synthase expression can be measured using procedures which are well known to persons skilled in the art. In the present application, effects on mRNA levels have been measured by Northern blot analysis and nuclear run-on transcription assay, and effects on the growth of human tumour cells have been measured by counting cell numbers using a Coulter counter.

Antisense oligonucleotides to thymidylate synthase may inhibit, stimulate or have no effect on thymidylate synthase expression. Of these, preferred antisense oligonucleotides are those which either inhibit or stimulate thymidylate synthase expression, and particularly preferred antisense oligonucleotides are those which inhibit thymidylate synthase expression.

By inhibition of thymidylate synthase expression we mean inhibition of at least 10% relative to the untreated control, measured at day 4 using the assay described in Example 1.2. Preferably inhibition of thymidylate synthase expression is at least 20% and most preferably inhibition is at least 40%.

By stimulation of thymidylate synthase expression we mean stimulation of at least 10% relative to the untreated control, when measured at day 7 using the assay described in Example 1.2. Preferably stimulation is at least 20% and most preferably stimulation is at least 40%.

Preferably, the antisense oligonucleotides are from about 8 to about 50 nucleotides in length, more preferably from about 12 to about 40 nucleotides in length and most preferably from about 16 to about 30 nucleotides in length.

Specific examples of sequences of antisense oligonucleotides which regulate thymidylate synthase activity are shown in Table 1. The regions of TS mRNA targeted by the oligonucleotides are shown in Figure 7.

Table 1:

ANTISENSE OLIGONUCLEOTIDE	SEQUENCE
OLIGO 83	TTAAGGATGTTGCCACTGGC
OLIGO 86	AATGGCTGTTTAGGGTGCTT
OLIGO 90	TGTGGCCGGCTCGGAGCTGC
OLIGO 91	GCGCCATGCCTGTGGCCGGC
OLIGO 92	GCGCCATGCCTGTGGCCGGC
OLIGO 93	CCCGCCCGCCGCGCCATGCC

It will be appreciated that the invention is not restricted merely to those specific antisense oligonucleotides which are disclosed in Table 1 above but encompass oligonucleotides of from about 8 to about 50 nucleotides in length which selectively inhibit or selectively enhance thymidylate synthase production and which are selected from those regions of the TS gene which are described hereinbefore.

Hybridisation of an antisense oligonucleotide to its target nucleic acid sequence is mediated by the formation of hydrogen bonds between complementary bases on each nucleic

acid strand. Hybridisation may occur between nucleic acid strands which have varying degrees of complementarity, depending on the hybridisation conditions employed. The term "specifically hybridisable" is used to describe an oligonucleotide which has a sufficient degree of complementarity to ensure stable, specific binding to its target sequence, whilst avoiding non-specific binding to non-target sequences.

Antisense oligonucleotides may be designed to hybridise to any region within the thymidylate synthase mRNA molecule, including the coding region, the 5'untranslated region, the 3'untranslated region, the 5'cap region, introns and intron/exon splice junctions.

Hybridisation of the antisense oligonucleotide to thymidylate synthase mRNA may affect any aspect of mRNA function, for example mRNA translocation, mRNA splicing, mRNA translation, or the feedback inhibition mechanism regulated by the binding of thymidylate synthase protein to binding sites within the thymidylate synthase mRNA molecule.

An oligonucleotide is a polymeric molecule which is assembled from nucleotide or nucleoside monomers. The monomers may consist of naturally occurring bases, sugars and inter-sugar linkages or may also contain non-naturally occurring derivatives which modify the properties of the oligonucleotide, for example, phosphorothiorated oligonucleotides have been used in the present application to increase resistance to nuclease degradation.

Preferred oligonucleotides may contain phosphorothiorates, phosphotriesters, methyl phosphonates or short chain alkyl, cycloalkyl or heteroatomic intersugar linkages. Other preferred oligonucleotides may contain a peptide nucleic acid backbone. Particularly preferred oligonucleotides are those containing phosphorothiorates (Summerton, J.E. and Weller, D.D., U.S. Patent No: 5,034,506).

The oligonucleotides may be manufactured using any convenient method of synthesis. Examples of such methods may be found in standard textbooks, for example "Protocols for Oligonucleotides and Analogues; Synthesis and Properties," Methods in Molecular Biology Series; Volume 20; Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993; 1st Edition.

In a further aspect of the present invention, there is provided a combination product comprising an antisense oligonucleotide targeted to thymidylate synthase in combination with an anticancer agent. The antisense oligonucleotide and the anticancer agent may be administered separately, sequentially, simultaneously or in a mixture.

The anticancer agent may cover three main categories of therapeutic agent:

(i) thymidylate synthase inhibitors such as Tomudex (N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl)-L-glutamic acid) (European Patent Application no. 0239362, Example 7, compound no. 8 therein); Zeneca development compound ZD9331 ((S)-2-(2-fluoro-4-[N-(4-hydroxy-2,7-dimethylquinazolin-6-ylmethyl)-N-(prop-2-ynyl)amino]benzamido)-4-(1H-1,2,3,4-tetrazol-5-yl)butyric acid) (European Patent Application no. 0562734, Example 3 thereof); LY 231514 (Eli Lilly Research Labs, Indianapolis, IN); 1843U89 (Glaxo-Wellcome, Research Triangle Park, NC); AG337 and AG331 (both by Agouron, La Jolla, CA) (Touroutoglou and Pazdur, Clin. Cancer Res., 2, 227-243, 1996).

(ii) cytostatic agents such as antioestrogens (for example tamoxifen, toremifene, raloxifene, droloxifene, iodoxyfene), progestogens (for example megestrol acetate), aromatase inhibitors (for example anastrozole, letrazole, vorazole, exemestane), antiprogestogens, antiandrogens (for example flutamide, nilutamide, bicalutamide, cyproterone acetate), LHRH agonists and antagonists (for example goserelin acetate, luprolide), inhibitors of testosterone 5α-dihydroreductase (for example finasteride), anti-invasion agents (for example metalloproteinase inhibitors like marimastat and inhibitors of urokinase plasminogen activator receptor function) and inhibitors of growth factor function, (such growth factors include for example EGF, FGFs, platelet derived growth factor and hepatocyte growth factor such inhibitors include growth factor antibodies, growth factor receptor antibodies, tyrosine kinase inhibitors and serine/threonine kinase inhibitors).

(iii) antiproliferative/antineoplastic drugs and combinations thereof, as used in medical oncology, such as antimetabolites (for example antifolates like methotrexate, fluoropyrimidines

like 5-fluorouracil, purine and adenosine analogues, cytosine arabinoside); antitumour antibiotics (for example anthracyclines like doxorubicin, daunomycin, epirubicin and idarubicin, mitomycin-C, dactinomycin, mithramycin); platinum derivatives (for example cisplatin, carboplatin); alkylating agents (for example nitrogen mustard, melphalan, chlorambucil, busulphan, cyclophosphamide, ifosfamide, nitrosoureas, thiotepa); antimitotic agents (for example vinca alkaloids like vincrisitine and taxoids like taxol, taxotere); topoisomerase inhibitors (for example epipodophyllotoxins like etoposide and teniposide, amsacrine, topotecan).

Particularly preferred anticancer agents are thymidylate synthase inhibitors such as Tomudex (N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl)-L-glutamic acid) (European Patent Application no. 0239362, Example 7, compound no. 8 therein) and the Zeneca development compound ZD9331 ((S)-2-(2-fluoro-4-[N-(4-hydroxy-2,7-dimethylquinazolin-6-ylmethyl)-N-(prop-2-ynyl)amino]benzamido)-4-(1H-1,2,3,4-tetrazol-5-yl)butyric acid) (European Patent Application no. 0562734, Example 3 thereof).

In a further aspect of the present invention there is provided a pharmaceutical composition comprising a combination product as defined hereinbefore and a pharmaceutically acceptable diluent or carrier.

The pharmaceutical composition may be in a form suitable for oral use, for example a tablet, capsule, aqueous or oily solution, suspension or emulsion; for topical use, for example a cream, ointment, gel or aqueous or oily solution or suspension; for nasal use, for example a snuff, nasal spray or nasal drops; for vaginal or rectal use, for example a suppository; for administration by inhalation, for example as a finely divided powder such as a dry powder, a microcrystalline form or a liquid aerosol; for sub-lingual or buccal use, for example a tablet or capsule; or particularly for parenteral use (including intravenous, subcutaneous, intramuscular, intravascular or infusion), for example a sterile aqueous or oily solution or suspension. In general the above compositions may be prepared in a conventional manner using conventional excipients.

Tomudex is conveniently administered to humans by intravenous injection of a sterile aqueous solution at a dose in the range, for example, of 1 to 4 mg/m^2 of body surface area once every three weeks, preferably at a dose of 3 mg/m^2 once every three weeks.

ZD 9331 is conveniently dosed to humans by oral administration of a solid dosage form or by intravenous injection of a sterile aqueous solution. The oral dosage form is conveniently administered to humans at a total dose in the range, for example, of about 1 to 100 mg/kg (i.e. abut 35 mg/m^2 to 3.5 g/m^2) every three weeks given by a continuous or an intermittent dosing schedule, for example a dosing schedule of a three week dosing cycle comprising daily doses on days 1 to 5 only followed by no further doses until the next dosing cycle or a dosing schedule of a four week dosing cycle comprising daily doses on days 1 to 14 only followed by no further doses until the next dosing cycle. Preferably the oral dosage form is administered to humans at a total dose in the range, for example, of about 1 to 30 mg/kg every three or four week dosing cycle. The sterile aqueous solution is conveniently administered intravenously to humans at a total dose of up to 100 mg/m^2 every three weeks given by a continuous or an intermittent dosing schedule, for example, a dosing schedule of one dose per three week dosing cycle, a dosing schedule of a three week dosing cycle comprising daily doses on days 1 to 5 only followed by no further doses until the next dosing cycle, a dosing schedule of a three week dosing cycle comprising doses on days 1 and 8 only followed by no further doses until the next dosing cycle or a dosing schedule of a three week dosing cycle comprising continuous infusion on days 1 to 5 followed by no further dosing until the next dosing cycle. Preferably the sterile aqueous solution is administered intravenously to humans at a total dose in the range, for example, of about 20 to 50 mg/m^2 every three weeks given by a continuous or an intermittent dosing schedule as illustrated hereinbefore.

The antisense oligonucleotide is conveniently administered to humans by intravenous injection of a sterile aqueous solution at a dose per dosing cycle in the range, for example, of 0.1μg to 1g, preferably at a dose of 1mg to 100mg.

The amount of active ingredient that is combined with one or more excipients to produce appropriate dosage forms will necessarily vary depending upon the particular

component of the combination product, the host treated and the particular route of administration. For example, a formulation intended for oral administration to humans will generally contain, for example, from 0.5μg to 2g of active agent compounded with appropriate and convenient amounts of excipients which may vary from about 5 to about 98 percent by weight of the total composition. A formulation intended for parenteral administration to humans will generally contain 0.1μg to 50mg. Dosage unit forms will generally contain about 1μg to about 500mg of an active ingredient.

In a further aspect of the invention there is provided a method for the treatment of cancer (or a method for providing an antiproliferative effect) which comprises administering to a warm-blooded animal an effective amount of a combination product as defined above. The invention also provides the use of such a combination product in the production of a new medicament for the treatment of cancer (or for the treatment of proliferative disease).

Abbreviations used in this application are set out below.

TS	thymidylate synthase
CMV	cytomegalovirus
5-FU	5-fluorouracil
PBS	phosphate-buffered saline
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
FBS	fetal bovine serum
MT	metallothionein
ODN	oligodeoxynucleotide
bp	base pairs
DMEM	Dulbecco's modified Eagle medium
oligo	oligonucleotide

The invention will now be illustrated but not limited by reference to the following Example and Figures wherein:

Figure 1 shows that MCF-7 cell growth is inhibited by transfection with antisense TS oligo 86 (targeted to the translation stop site), but is enhanced by transfection with antisense TS oligos 90 or 92 (targeted to sequences at or near the translation start site).

Figure 2 shows that HeLa cell growth is inhibited by transfection with antisense TS oligo 86 (targeted to the translation stop site), but is enhanced after transfection with antisense TS oligo 91 (targeted to the translation start site).

Figure 3 shows that HeLa cell growth is inhibited by transfection with antisense TS oligo 83 (targeted to a 3' untranslated sequence downstream of the translation stop site), but is not affected by transfection with antisense TS oligo 81 (targeted to the translation start site).

Figure 4 shows that transient transfection of HeLa cells with oligo 86 (targeted to the TS translation stop site) enhances sensitivity to Tomudex and that oligo 91 (targeted to the TS translation start site) reduces sensitivity to Tomudex.

Figure 5 shows that transient transfection of HeLa cells with oligo 83 (targeted to a sequence in the 3' untranslated region of TS mRNA) enhances sensitivity to Tomudex whereas oligo 81 (targeted to a 3' sequence downstream of that targeted by oligo 81) has no effect on Tomudex sensitivity.

Figure 6 shows that antisense TS oligo 91, but not oligo 86, stimulates TS gene transcription in human HeLa cells.

Figure 7 shows the sequence of human mRNA for thymidylate synthase (EC 2.1.1.45), bases 1 to 1536.

Example 1

Example 1.1: Experimental Methods

Cell culture:

MCF-7 (human breast adenocarcinoma) cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 10 mM Hepes (pH 7.4) and 0.1% Gentamycin.

Vector construction:

Expression vectors pAS/TSS and pAS/exon1,2 were designed to produce, upon transfection into MCF-7 cells, single-stranded antisense RNA molecules containing double-stranded 30 bp oligonucleotides complementary to the TS mRNA at one of two sites. Oligodeoxynucleotides corresponding to each strand of the human TS cDNA at positions 111 to 140 (pAS/TSS; targeting a 30 bp region adjacent to, and 2 bp away from, the translation start site) or 296 to 325 (pAS/exon1,2; targeting a 30 bp region spanning the exon 1/exon 2 boundary) were synthesized. Numbering of bases was according to GenBank accession no. X02308 (Takeishi *et al.*, 1985). In order to facilitate cloning, six additional nucleotides were incorporated (5 at the 5' end, 1 at the 3' end) of each oligodeoxynucleotide to produce *Hind* III or *Xba* I sticky ends when complementary strands were annealed. Single-stranded oligonucleotides (4 mg each) were annealed in 3X SET (450 mM NaCl, 60 mM Tris-HCl, 3 mM EDTA, pH 7.8) by treating the mixture for 5 min at 90°C, 5 h at 50°C, then 16 h at 25°C. Double-stranded products were identified by gel electrophoresis, and directionally inserted into the *Hind* III and *Xba* I sites of pRC/CMV (Invitrogen Corp., San Diego, CA). The orientation of cloning was confirmed by direct sequencing.

Oligodeoxynucleotides:

Fully phosphorothioated 20-mer oligonucleotides (ODNs) were synthesized by Isis Pharmaceuticals (Carlsbad, CA). The 6 terminal nucleotides at the 5' and 3' ends of each ODN were 2'-methoxyethoxy-modified to make them resistant to intracellular nucleases and increase their stability within cells (M^{c}Kay *et al.*, 1996), but the internal 8 nucleotides without methoxyethoxy groups were susceptible to RNase H cleavage. Oligo 86 was complementary to

mRNA from base positions 1035 to 1054 (GenBank accession no. X02308; Takeishi *et al.*, 1985), which surround the TS mRNA translation stop site (UAG at bases 1045 to 1047). Oligo 90 was complementary to base positions 111 to 130, which are 3' and proximal to the translation start site (AUG at bases 106 to 109). Oligo 92 was complementary to base positions 101 to 120, including and surrounding the translation start site.

Transfection:

Antisense RNA expression vectors were transfected into MCF-7 cells using Lipofectamine (GIBCO BRL, Burlington, ON, Canada), a polycationic liposome formulation. 1.5 X 10^6 cells were dispensed into 100 X 15 mm tissue culture plates and allowed to adhere overnight. Cells were washed once with 3 ml OptiMEM (GIBCO BRL, Burlington, ON, Canada), followed by exposure to 6 ml of a mixture of double-stranded expression vector DNA (0.83 μg/ml) plus Lipofectamine (1.67 μg/ml) in opti-MEM for 6 h at 37°C in a 5% CO_2 incubator. The DNA/Lipofectamine mixture was removed and replaced with 10 ml DMEM with supplements. Control cells were transfected with pRC/CMV vector without insert. Transiently-transfected cells were used within 1 to 6 days. Stably-transfected cells were allowed to recover in complete non-selective medium without Geneticin for 48 h, then grown in the presence of Geneticin (400 μg per ml, active form [Gibco/BRL]) to allow selection of colonies harboring pRC/CMV control vectors, or pRC/CMV vectors expressing RNA complementary to sequences surrounding the translation start site (pAS/TSS).

Single-stranded oligodeoxynucleotides (0.5 μM or 1.0 μM) were transiently transfected into 1.5 X 10^6 MCF-7 cells adhering to 100 X 15 mm tissue culture plates, in a total volume of 5 ml in the presence of Lipofectamine (2 μg/ml) in Opti-MEM. Control cells were treated with Lipofectamine/Opti-MEM without added DNA. Cells were washed after 6 h, medium plus supplements added as described above, and then grown for 48 hours before isolation of nuclei for run-on transcription measurement.

Southern blot analysis:

Total DNA was isolated from pAS/TSS transfected cells as follows. Cells were incubated in lysis buffer (50 mM Tris-HCl [pH8.0], 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate, 0.5 mg/ml proteinase K) for 6 h at 55°C. One-third the volume of 6 M NaCl was

added to precipitate non-nucleic acids by centrifugation at 10,000 X *g* for 15 min. The DNA i the supernatant was precipitated in isopropanol and washed with 70% ethanol. DNA was cleaved with *Hind* III for 16 h and analysed by Southern blotting (Sambrook *et al.*, 1989). Blots were hybridized with an [α-^{32}P]dCTP random primer-labeled pAS/TSS probe (Church and Gilbert, 1984), and were exposed to a phosphor screen and quantitated using a PhosphorImager and the ImageQuant program (Molecular Dynamics, Sunnyvale, CA). The nylon membranes were stripped and rehybridized with an *Alu* probe (300 bp of a human *Alu* restriction fragment inserted into pBR322 [Jelinek *et al.*, 1980]) in order to quantitate the amount of human DNA loaded in each lane (Koropatnick *et al.*, 1988).

Northern blot analysis:

RNA was isolated using RNeasy columns (Qiagen Inc., Chatsworth, CA) from cells transfected with the pAS/TSS expression vector. Ten or 15 ug of RNA per lane were separated on a 1.4% formaldehyde gel (Sambrook *et al.*, 1989) and transferred to a Hybond-N nylon membrane. Membranes were hybridized (Church and Gilbert, 1984) with either a pAS/TSS-generated riboprobe (Promega Corp., Madison, WI) designed to bind to antisense RNA, or a random primer-labeled, 1.9 kb *Xho* I fragment from pcHTS-1 (a eukaryotic expression vector containing the human TS cDNA: a generous gift from Dr. K. Takeishi, University of Shizuoka, Shizuoka, Japan)(Takeishi *et al.*, 1985). Blots were stripped and rehybridized with cDNA probes to detect 18S ribosomal RNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Images were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Isolation of nuclei:

Relative transcription rates were determined by a nuclear run-on assay (Koropatnick *et al.*, 1997), a modification of the methods of Kikuchi *et al.* (1992) and Almendral *et al.* (1988). Nascent transcripts were extended *in vitro* (Marzluff and Huang, 1984) in parallel reactions using MCF-7 cell nuclei isolated 48 h following transient transfection with control or antisense TS RNA expression vectors, or single-stranded antisense TS oligodeoxynucleotides (or Lipofectamine alone), and from cells stably transfected with antisense RNA expression vectors. Adherent cells were rinsed twice with ice-cold PBS, scraped off with a rubber policeman, pelleted in PBS (5 min, 500 X *g*) and lysed by incubating 5 min at 4° C in 4 ml of lysis buffer (10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40). All subsequent steps were

rried out at 4° C. Complete cell lysis and integrity of released nuclei was checked by light microscopy, and nuclei were pelleted by centrifugation at 500X*g* for 5 min. Nuclei were then resuspended in 4 ml of lysis buffer by vortexing, pelleted by centrifugation, resuspended in 200 µl of nuclei storage buffer (40% glycerol, 5 mM $MgCl_2$, 50 mM Tris-HCl [pH 8.0], 0.1 mM EDTA) in a 15 ml conical polypropylene centrifuge tube, and immediately frozen in liquid nitrogen and stored at -120° C until use up to one month later.

Run-on transcription:

RNA elongation reactions were performed for 30 min at 30°C using 2 X 10^7 nuclei/400 µl reaction. Reaction mixtures were composed of 200 µl nuclei storage buffer plus 200 µl of sterile 2X reaction buffer (10 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 0.3 M KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, 5 mM dithiothreitol, and 2 µl [α-^{32}P]UTP or [α-^{32}P]CTP [≈ 3000 Ci/mmole, 10 mCi/ml]). Nucleotides, radionucleotides, and dithiothreitol were added immediately prior to use. Nascent RNA transcripts were allowed to elongate for 30 min at 30°C on a shaking platform, followed by addition of 600 µl of RNase-free DNase I (0.04 units RQ1 DNase I [RNase-free; Promega Corp.], 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Tris-HCl [pH 7.4]). The ^{32}P-labeled RNA was isolated using Trizol (Gibco/BRL), and the final precipitated RNA was dissolved in Church hybridization buffer (1 mM EDTA, 0.5 M $NaHPO_4$ [pH 7.2], 7% sodium lauryl sulphate [SLS]), to a final concentration of 4 X 10^6 cpm per ml .

Hybridization of radiolabeled RNA to immobilized unlabeled probes:

In order to distinguish between TS sense and antisense RNA molecules produced in isolated nuclei from transfected cells, target DNA (immobilized on nitrocellulose filters in triplicate dots, 2 µg per dot) consisted of single-stranded synthetic oligonucleotides rather than TS cDNA. Strand-specific oligonucleotide probes were also used to assess levels of human metallothionein-2 (MT-2) mRNA and antisense RNA as positive and negative controls, respectively. Single-stranded oligonucleotides were immobilized on nitrocellulose filters by dissolving in 6XSSC (16 µg per ml) and applying 125 µl per dot using a BioRad dot-blot apparatus. Unlabeled complementary cDNA probes for GAPDH mRNA (Denhardt *et al.*, 1988) and 18S ribosomal RNA (Behrend *et al.*, 1994) were denatured and immobilized on the same nitrocellulose filters (5 µg per dot, triplicate dots) using a previously-described protocol (Koropatnick, 1988). Hybridization of radiolabeled RNA to these dots assessed transcription of

GAPDH and 18S rRNA genes, and acted as internal standards against which to measure chan in TS gene transcription. For cells transfected with single-stranded oligonucleotides, TS gene transcription was assessed by hybridization of radiolabeled TS RNA transcripts to immobilized target DNA consisting of a 1.9 kb *Xho I* fragment isolated from pCHTS-1.

Nitrocellulose filters containing triplicate dots of oligonucleotide and cDNA probes to assess run-on transcription of antisense TS RNA expression vectors, and endogenous TS, MT-2,GAPDH and 18S rRNA genes, were prehybridized in Church buffer for 20 min at 65° C in a Hybaid hybridization chamber. The prehybridization buffer was then removed, 2 mls of radiolabeled RNA resulting from 30 min of run-on transcription in isolated nuclei (in Church hybridization buffer, 4 X 10^6 cpm per ml) was added, and the filters were hybridized for 48 h at 65° C. The filters were then washed twice at 65°C in posthybridization buffer (40 mM Na_2HPO_4, 1% SDS; 20 min per wash). Posthybridization buffer was removed and 8 ml of RNase A (1 µg per ml in 6XSSC) was added and incubated for 30 min at 37° C to reduce signal from unhybridized radiolabeled RNA. After a final wash in posthybridization buffer (10 min, 37° C) filters were blotted dry and bound radioactivity visualized and quantitated using a a phosphorimager and the ImageQuant program (Molecular Dynamics, Sunnyvale, CA). Relative transcription of antisense TS expression vectors, endogenous TS genes, and MT-2 genes was defined as:

$$\text{Relative transcription rate} = \frac{\text{(hybridisation signal from gene of interest)}}{\text{(hybridisation signal for GAPDH or 18S rRNA genes)}}$$

TS oligonucleotide probes:

Bases in bold-face (below) form part of restriction endonuclease sites, and are not sense or antisense TS sequences. Numbering indicates the distance from the beginning of the transcription start site.

TS cDNA nucleotides 111 to 140

sense TS (JK-5): **CTAGA**TGTGGCCGGCTCGGAGCTGCCGCGCCGGCCA

tisense TS (JK-2): **AGCTT**GGCCGGCGCGGCAGCTCCGAGCCGGCCACAT

TS cDNA nucleotides 296 to 325

sense TS (JK-3): **CTAGA**GCTACAGCCTGAGAGATGAATTCCCTCTGCA

antisense TS (JK-4): **AGCTT**GCAGAGGGAATTCATCTCTCAGGCTGTAGCT

MT-2 oligonucleotide probes (Karin and Richards, 1982):

Sense and antisense oligonucleotide sequences did not have non-complementary sequences added to the 5' and 3' ends. Numbering indicates the distance from the translation start site.

MT cDNA nucleotides -14 to 6

sense MT: CTCTTCAGCACGCCATGGAT

MT cDNA nucleotides 204 to 223

antisense MT: AGGGTCTACCTTTCTTGCGC

Example 1.2: Antisense oligodeoxynucleotide targeting regions at or near the translation stop site at the 3' end of the TS gene as a method to inhibit growth of human tumour cells

a) A 20-mer antisense oligodeoxynucleotide (oligo 86) targeted to the translation stop site at the 3' end of the thymidylate synthase mRNA is growth inhibitory (cytostatic) in a human breast cancer cell line (MCF-7 cells). Antisense oligonucleotides of the same length (oligos 90 and 92), targeted to regions at or near the translation start site at the 5' end of the TS mRNA, are not cytostatic **(Figure 1)**.

b) A 20-mer antisense oligodeoxynucleotides targeted to the TS mRNA translation start site (oligos 91 and 93) did not inhibit growth of a human cervical carcinoma (HeLa) cell line. In fact, growth was significantly enhanced. Antisense oligodeoxynucleotides targeted to the 3' end of the TS mRNA, including the translation stop site (oligo 86) or a sequence in the 3' untranslated region (oligo 83) significantly inhibited HeLa cell growth. An antisense TS

oligonucleotide targeted to another sequence in the 3' untranslated region of TS mRNA (ol 81) had no effect on HeLa cell growth **(Figures 2 and 3)**.

Therefore, no antisense TS oligodeoxynucleotides targeted to the translation start site were successful in inhibiting growth of two different human tumour cell lines (human breast carcinoma MCF-7 cells or human cervical carcinoma HeLa cells). **Two separate antisense TS oligodeoxynucleotides targeted to the 3' end of the TS gene were potent inhibitors of human tumour cell growth.**

Example 1.3:

(a) Antisense oligodeoxynucleotide targeting of the thymidylate synthase translation stop site as a method to enhance human tumour cell sensitivity to the toxic effects of Tomudex (ZD 1694).

Antisense TS oligodeoxynucleotides (oligos 86 and 83) targeting sequences in the 3' untranslated region of TS mRNA enhanced human cervical carcinoma cell sensitivity to Tomudex. The enhancement in sensitivity was in addition to the directly cytostatic effects of oligos 86 and 83 **(Figures 4 and 5)**.

(b) Antisense oligodeoxynucleotide targeting of the thymidylate synthase translation start site as a method to enhance human cell resistance to the toxic effects of Tomudex (ZD 1694).

An antisense TS oligodeoxynucleotide (oligo 91) targeting the translation start site of TS mRNA enhanced human cervical carcinoma cell resistance to the toxic effects of Tomudex **(Figure 4)**.

Example 1.4: Induction of transcription of genes targeted with antisense nucleic acids as a screening method to identify appropriate target sequences for antisense nucleic acids.

Human tumour cells appear to compensate for antisense inactivation of specific mRNA by increasing transcription of genes producing the target sequences, a process that can be termed "compensatory transcription", resulting in resistance to the effectiveness of antisense nucleic acids. It has been observed, that TS gene transcription is induced in human MCF-7 breast carcinoma cells by treatment with antisense TS RNA and oligodeoxynucleotides targeted to regions at or near the TS mRNA translation start site. The same phenomenon has been observed in human HeLa cells transiently-transfected with antisense TS oligo 91 (targeted to the translation start site), but not in response to oligo 86 (targeted to the translation stop site) **(Figure 6)**. Increased specific gene transcription in response to transfected antisense nucleic acids would indicate that the target sequence is inappropriate to achieve downregulated gene expression. On the other hand, it may be an appropriate sequence to target to achieve upregulated gene expression (to increase resistance to chemotherapeutic drugs, for example, in normal tissues.

In summary, the present invention has demonstrated that antisense oligonucleotides, targeted against selected regions of thymidylate synthase mRNA, can effectively inhibit growth when administered alone. They can also enhance cell killing by Tomudex. Conversely, antisense oligonucleotides targeted to certain mRNA regions (for example, the translation start site) may either be ineffective, or enhance growth and survival during exposure to Tomudex. Ineffectiveness may be due to oligonucleotide-induced TS gene transcription. It is essential to identify TS mRNA regions that may be effectively targeted with antisense sequences to inhibit tumour cell growth and enhance the toxicity of anticancer drugs. Furthermore, the mechanism by which antisense sequences targeted to 5' TS mRNA regions induce TS gene transcription has important implications for choosing antisense targets in TS mRNA in particular, and for optimising antisense strategies in general.

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Figures
1/7

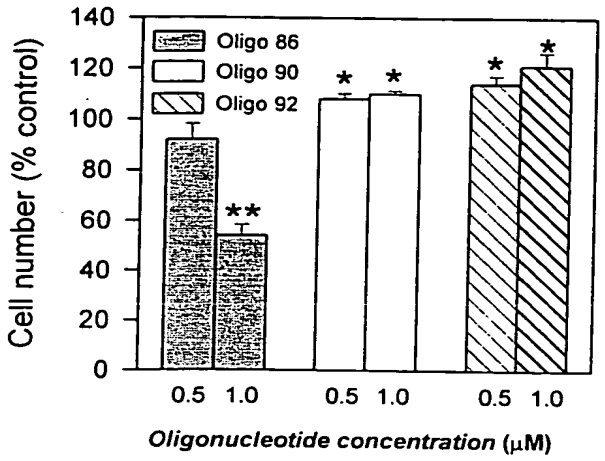
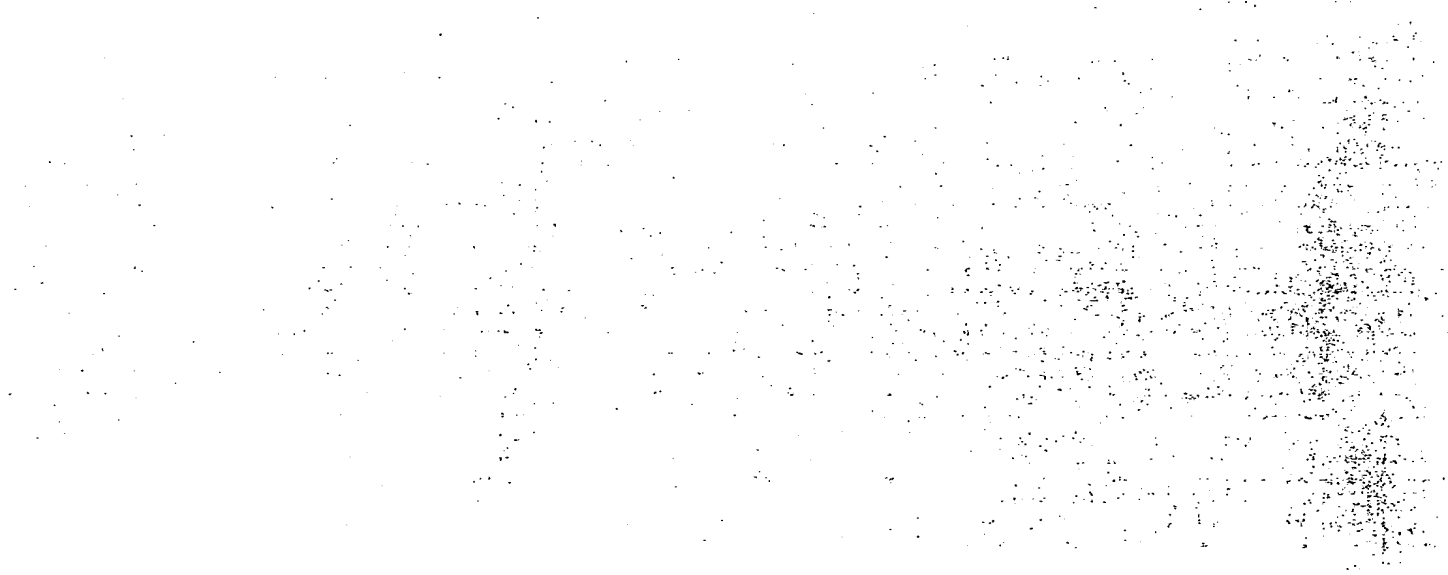


Figure 1: MCF-7 cell growth is inhibited by transfection with antisense TS oligo 86 (targeted to the translation stop site), but is enhanced by transfection with antisense TS oligos 90 or 92 (targeted to sequences at or near the translation start site).

Cells were transiently-transfected with 0.5 or 1.0 μM antisense TS oligonucleotides in Lipofectin as described. Cell numbers were measured by Coulter counter in triplicate flasks after 4 days of growth. Control cells were treated with Lipofectin without oligonucleotides. Cell growth is expressed as a percentage of growth of control cells.

*: Significantly higher than control ($p<0.05$, one way analysis of variants).

**: Significantly lower than control ($p<0.05$, one way analysis of variants).



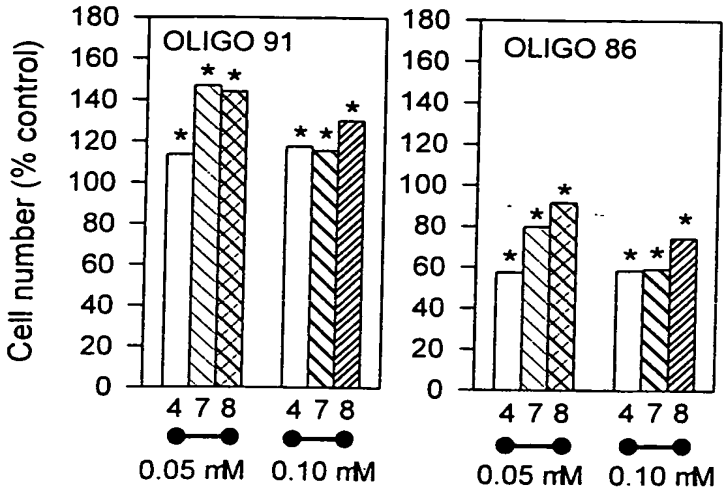
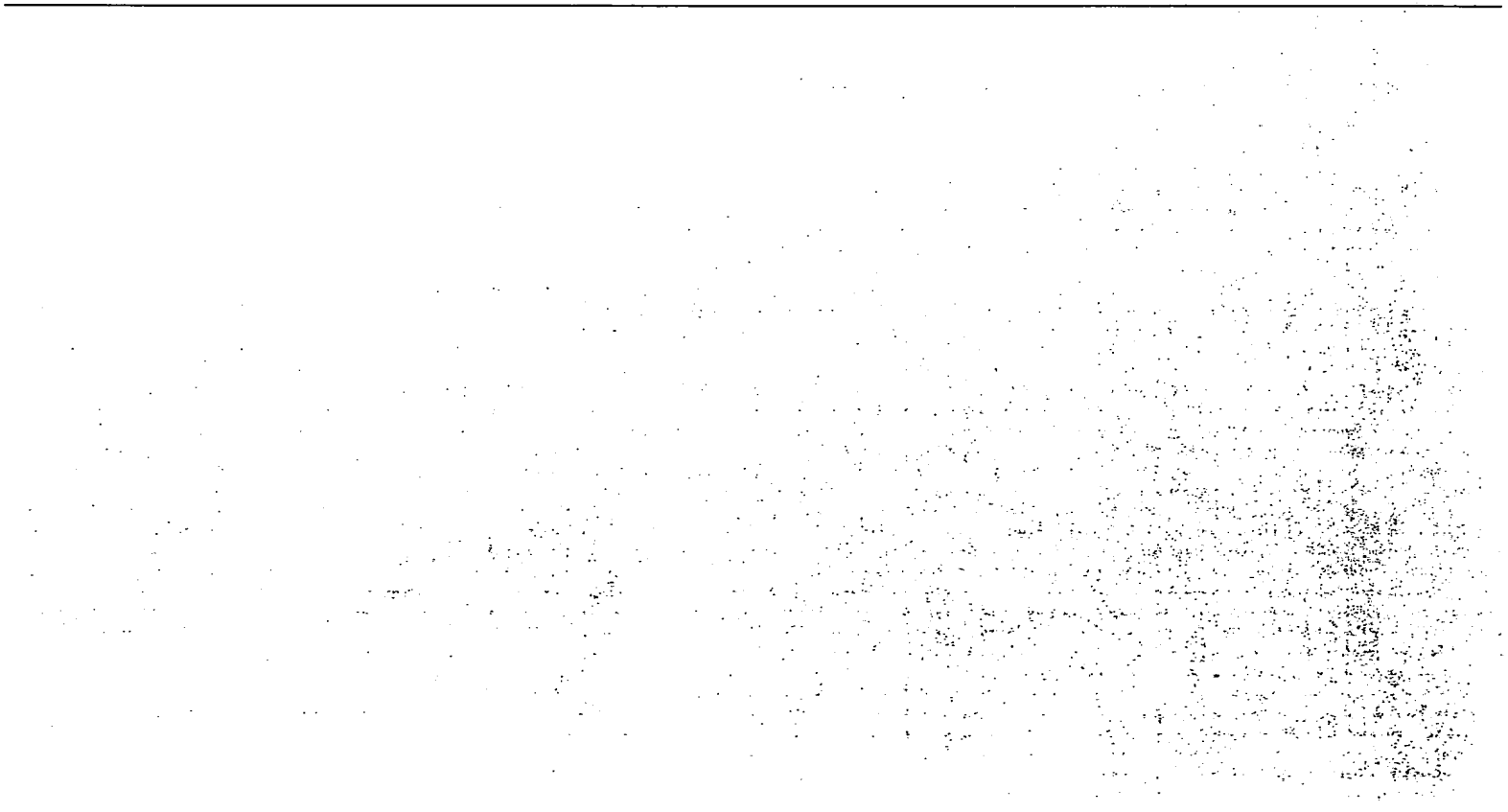


Figure 2: HeLa cell growth is inhibited by transfection with antisense TS oligo 86 (targeted to the translation stop site), but is enhanced after transfection with antisense TS oligo 91 (targeted to the translation start site).

HeLa cells were transfected with 0.05 or 0.10 μM antisense TS oligonucleotides in Lipofectin (10 μg/ml) for 4 hours as described. Note that oligo concentrations are considerably lower than those used for MCF-7 cells. The efficiency of Lipofectin-mediated DNA transfection of HeLa cells is greater than for MCF-7 cells. Lipofectin was removed, the cells were trypsinised, and 25,000 viable cells per flask were plated in tissue culture flasks. Cell numbers were measured by Coulter counter in triplicate flasks after 4, 7 and 8 days of growth. Control cells were treated with Lipofectin without oligonucleotides. Cell growth is expressed as a percentage of growth of control cells.

*: Significantly higher than control ($p<0.05$, Student's t-test).

**: Significantly lower than control (Student's t-test).



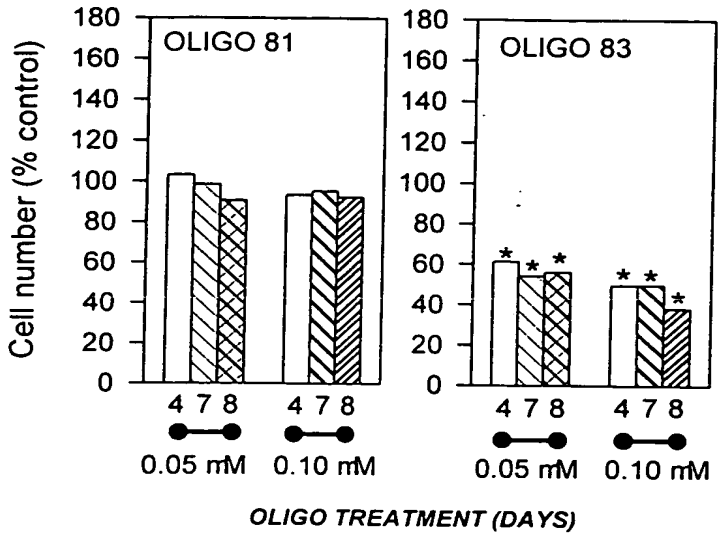
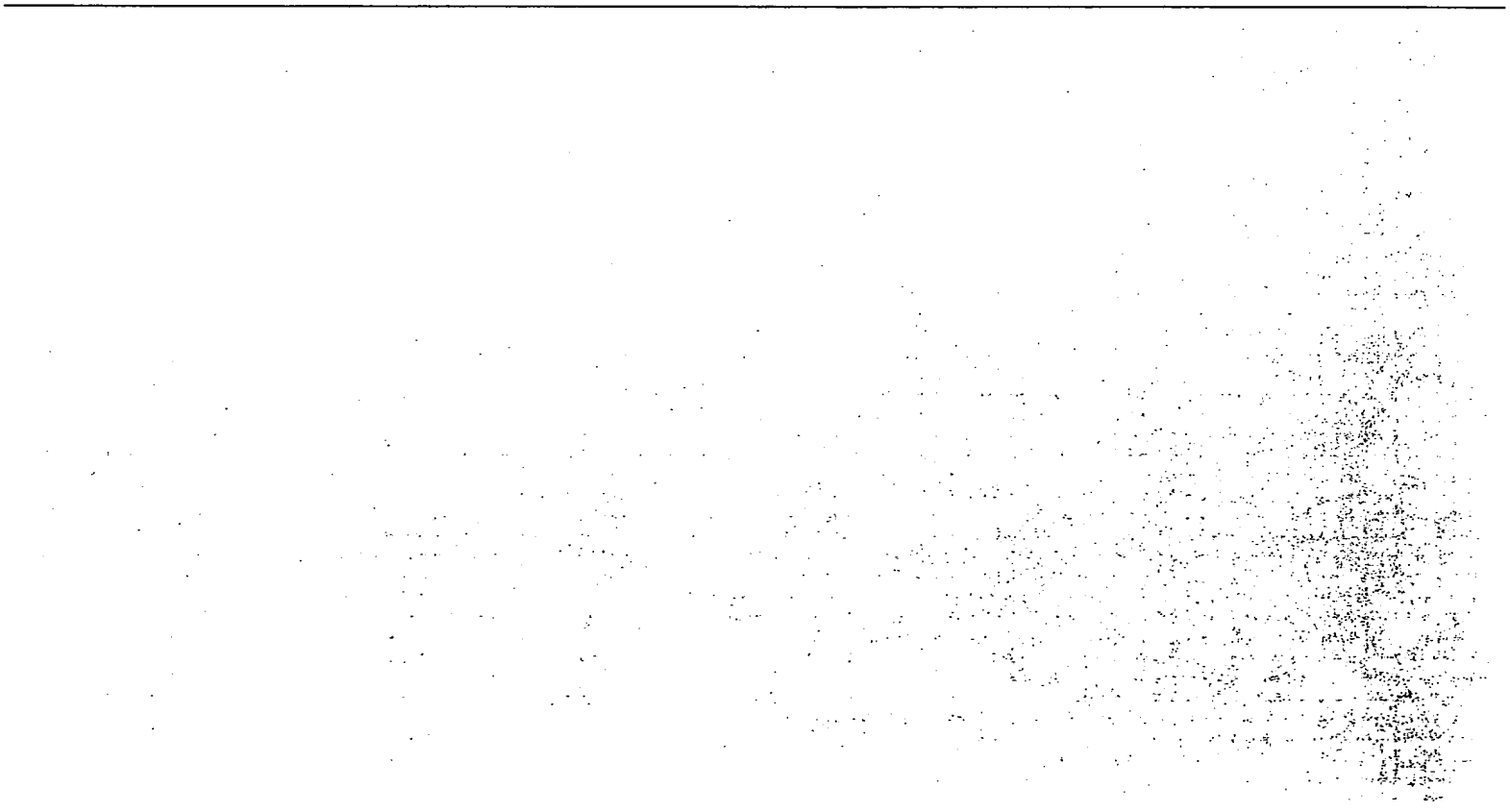


Figure 3: HeLa cell growth is inhibited by transfection with antisense TS oligo 83 (targeted to a 3' untranslated sequence downstream of the translation stop site), but is not affected by transfection with antisense TS oligo 81 (targeted to the translation start site).

The experimental protocol was as described in the legend to Figure 2.



Figures
4/7

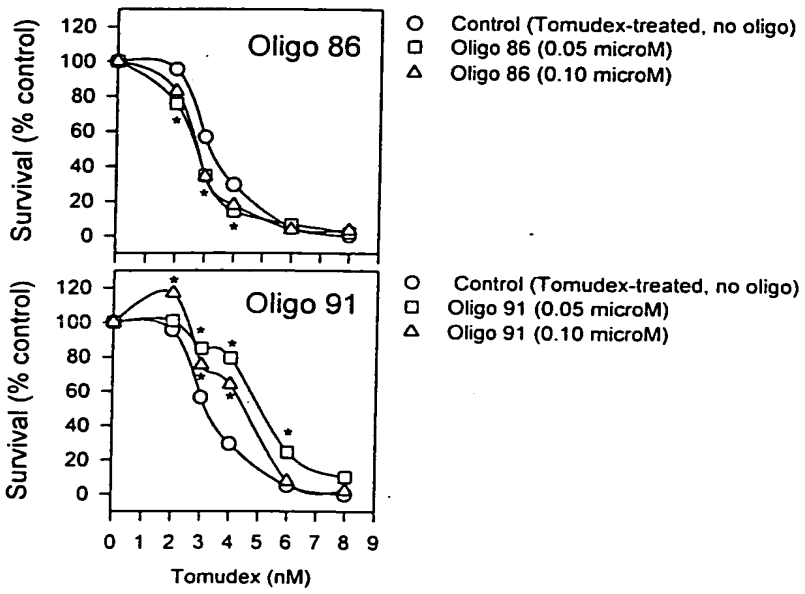
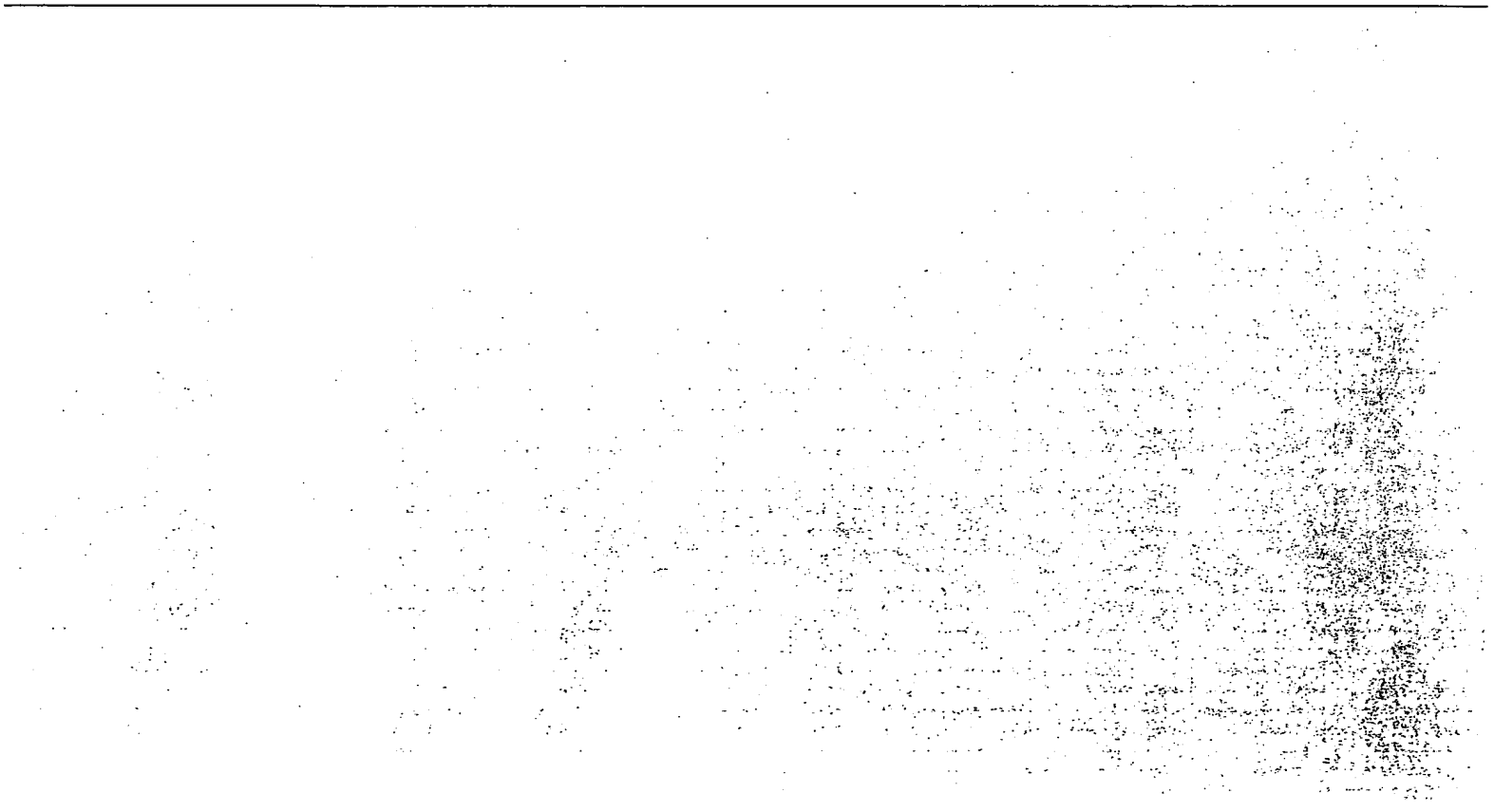


Figure 4: Transient transfection of HeLa cells with oligo 86 (targeted to the TS translation stop site) enhances sensitivity to Tomudex. Oligo 91 (targeted to the TS translation start site) reduces sensitivity to Tomudex.

HeLa cells were transfected with 0.05 and 0.10 μM antisense TS oligonucleotides and plated in flasks at low density, as described for Figure 2. Tomudex (0-8 nM) was added (triplicate flasks for each Tomudex concentration) and the cells allowed to proliferate for 7 days. Cell numbers were measured by Coulter counting at that time. Survival is plotted as a percentage of growth in cells transfected with oligonucleotide, but untreated with Tomudex. Therefore, these data reveal inhibition or enhancement of Tomudex killing independent of growth inhibition or enhancement induced by oligonucleotides in the absence of Tomudex. The mean of three values is plotted. Error bars were smaller than the size of the symbol in each case.

*: Significantly different from control ($p<0.05$, Student's t-test).



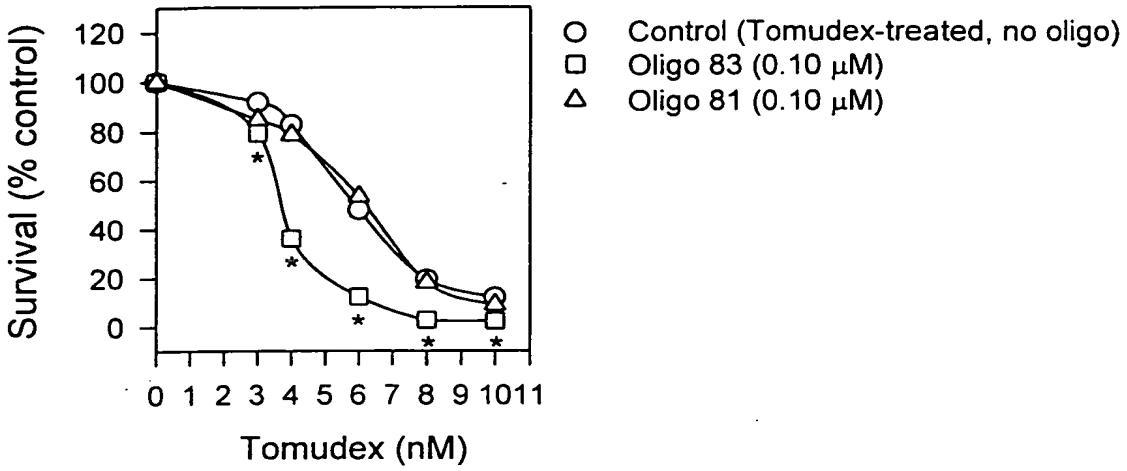
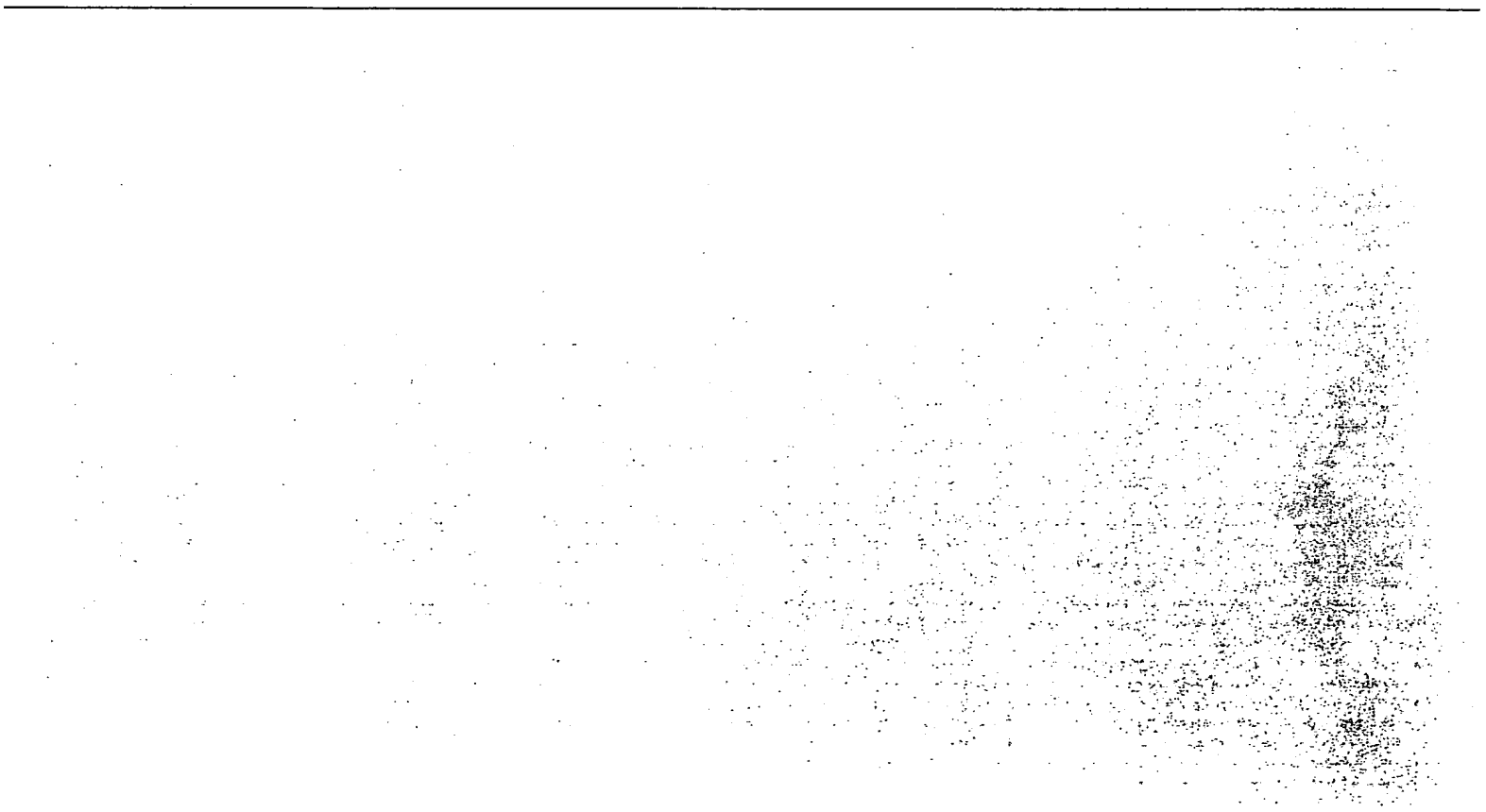


Figure 5: Transient transfection of HeLa cells with oligo 83 (targeted to a sequence in the 3' untranslated region of TS mRNA) enhances sensitivity to Tomudex. Oligo 81 (targeted to a 3' sequence downstream of that targeted by oligo 81) has no effect on Tomudex sensitivity.

HeLa cells were transfected with 0.10 μM antisense TS oligonucleotides and plated in flasks at low density, as described for Figure 2. Tomudex (0-10 nM) was added (triplicate flasks for each Tomudex concentration) and the cells allowed to proliferate for 4 days. Cell numbers were measured by Coulter counting at that time. Survival is plotted as a percentage of growth in cells transfected with oligonucleotide, but untreated with Tomudex. Therefore, these data reveal enhancement of Tomudex killing independent of growth inhibition induced by oligonucleotides in the absence of Tomudex. The mean of three values is plotted. Error bars were smaller than the size of the symbol in each case.

*: Significantly different from control ($p<0.05$, Student's t-test).



Figures

6/7

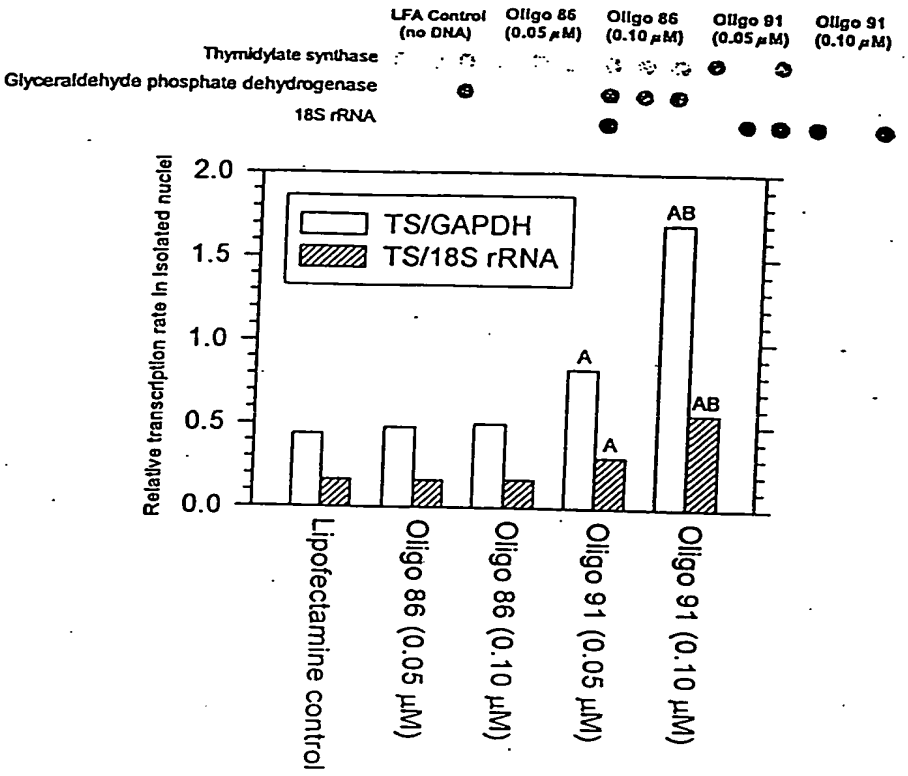


Figure 6: Antisense TS oligo 91, but not oligo 86, stimulates TS gene transcription in human HeLa cells.

The same HeLa cells for which data are presented in Figure 2 were assessed for run-on transcription of TS, glyceraldehyde phosphate dehydrogenase (GAPDH), and 18S rRNA genes. Briefly, cells were transfected with 0.05 and 0.10 μM antisense TS oligonucleotides in Lipofectin (10 μg/ml), or with Lipofectin alone (LFA control) for 4 hours as described in the progress report. Lipofectin was removed and cells were trypsinised and replated in tissue culture flasks. Four days after transfection, nuclei were isolated from approximately $5x10^6$ cells for each treatment and initiated TS, GAPDH, and 18S rRNA transcripts allowed to incorporate [^{32}P]-CTP for 30 minutes. Alcohol-precipitable radiolabeled RNA was hybridised for 48 hours to unlabeled TS, GAPDH, and 18S rRNA cDNA immobilised in triplicate dots on nylon membrane as described. Relative transcription rate is presented as:

$$\text{Relative transcription rate} = \frac{\text{(hybridisation signal from gene of interest)}}{\text{(hybridisation signal for GAPDH or 18S rRNA genes)}}$$

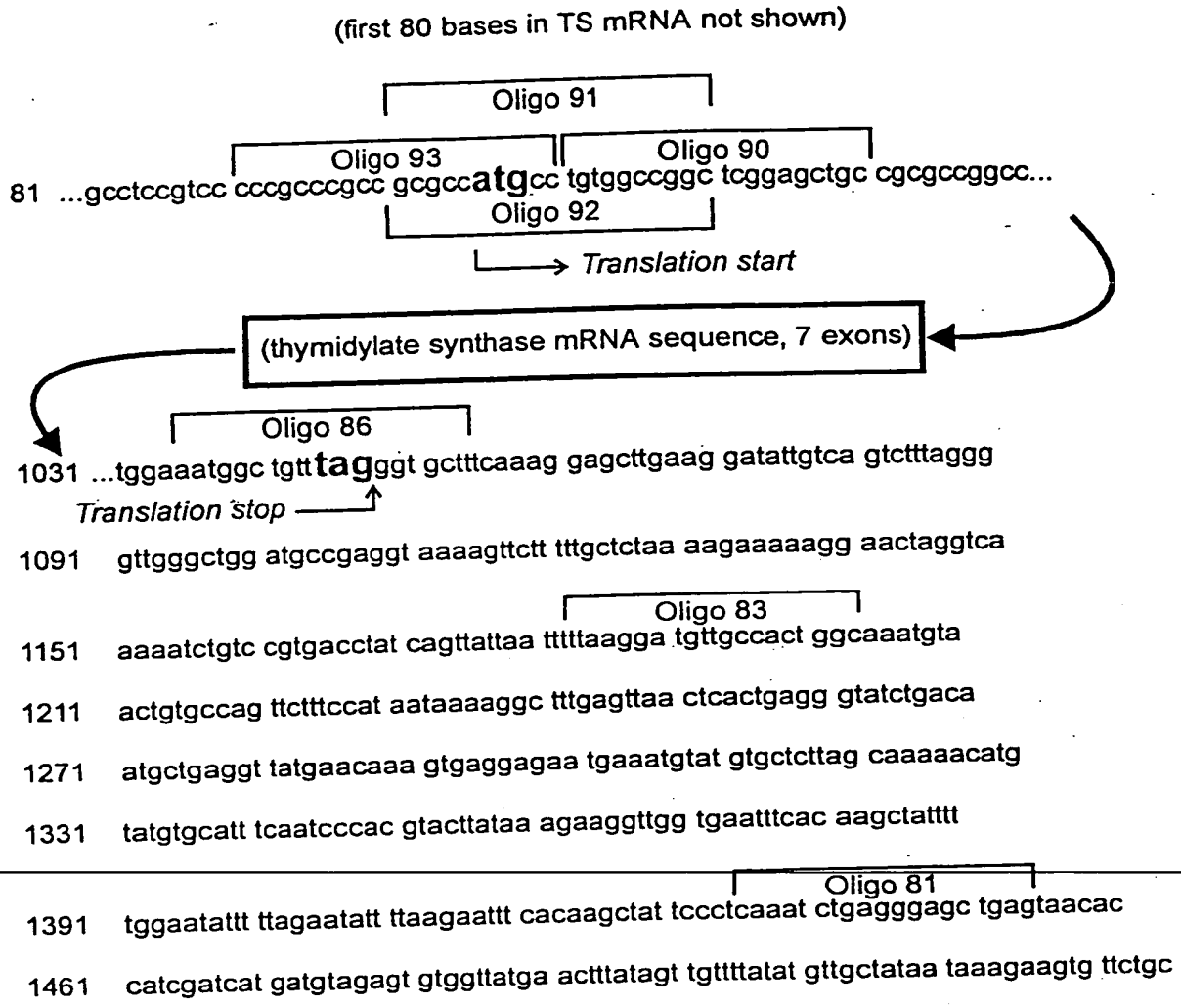


Figure 7: Human mRNA for thymidylate synthase (EC 2.1.1.45), bases 1 to 1536.

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